Molecular biomarkers in urothelial carcinoma of the bladder: are we there yet?

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Abstract | The unprecedented advances in cancer genetics and genomics are rapidly affecting the clinical management of solid tumors. Molecular diagnostics are now an integral part of routine clinical management for patients with lung, colon, and breast cancer. In sharp contrast, molecular biomarkers have been largely excluded from current management algorithms for urologic malignancies. The need for new treatment options that can improve upon the modest outcomes currently associated with muscle-invasive bladder cancer is evident, and validated prognostic molecular biomarkers that can help clinicians to identify patients in need of early, aggressive management are lacking. Robust predictive biomarkers that are able to forecast and stratify responses to emerging targeted therapies are also needed.

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Introduction

Accumulating molecular genetic evidence supports the existence of two broadly distinct pathogenetic pathways for bladder cancer development that parallel the contrasting biological and clinical phenotypes of non-muscle-invasive (superficial) and muscle-invasive urothelial carcinoma. Whereas the majority of invasive urothelial carcinomas are likely to originate from the progression of dysplasia to flat carcinoma *in situ* (CIS) and high-grade noninvasive lesions, superficial urothelial lesions are thought to develop from benign urothelium through a process of urothelial hyperplasia (Figure 1). A small percentage (10–15%) of all noninvasive lesions will progress to muscle-invasive disease, and genetic instability is crucial to this progression.[1–4]

Alterations in the Ras–MAPK and PI3K–AKT–mTOR pathways are largely responsible for promoting cell growth in urothelial neoplasia. Activating mutations in the genes encoding the Ras subfamily lead to activation of MAPK and PI3K pathways. Mo *et al.*[5] have proposed a model to explain the biomolecular process underlying early oncogenesis in noninvasive papillary urothelial neoplasms (Figure 2). In this model, Ras promotes survival and angiogenesis by activating the PI3K–AKT–mTOR and JAK–STAT pathways. Inactivation of the tumor suppressor gene *PTEN*—an mTOR pathway inhibitor—can also lead to activation of the AKT proteins and the JAK–STAT pathway. Alternatively, *FGFR3* mutations can trigger Ras activation, leading to a similar downstream signal transduction. *PIK3CA* and *FGFR3* mutations generally occur together, and there is evidence to suggest these mutations have an additive oncogenic effect.

Competing interests
The author declares no competing interests.

Emerging biomarkers in urothelial cancer

As our understanding of the molecular pathways involved in urothelial oncogenesis expands, advances in molecular prognostication, theranostics, and targeted therapy for bladder cancer sharply gain momentum.[6–24] Prognostic parameters that can accurately predict progression of non-muscle-invasive bladder cancer (NMIBC) are actively sought in order to identify patients in need of vigilant surveillance and aggressive treatment. The former is especially pertinent, as surveillance imposes a substantial financial burden and reduction in quality of life for patients with bladder cancer. In the USA, bladder cancer costs more to treat per patient than any other single solid tumor, with an estimated annual cost of $3 billion.[10] Given the poor outcomes currently associated with muscle-invasive disease (overall survival rate of 60% or less), markers that can improve prognostic accuracy in this group of patients are urgently needed.[25]

Chromosomal alterations and FISH assays

Alterations in chromosome 9 are the earliest genetic changes thought to occur in both of the divergent pathways of bladder cancer development. They are responsible for providing the necessary milieu of genetic instability that allows genetic defects to accumulate.[4] Several additional structural and numerical somatic chromosomal alterations are also common in bladder cancer. Chromosome 3q, 7p and 17q gains and 9p21 deletions (p16 locus) are of particular interest, given their potential diagnostic and prognostic value.[26,27]

Based on these numerical chromosomal alterations, a fluorescence *in situ* hybridization (FISH) cytogenetic urine assay has been developed, and this is now commercially available and widely used in clinical management.[28] Although it was initially only approved by the FDA for the surveillance of patients with previously diagnosed bladder cancer, the test has subsequently

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Key points

- Molecular genetic evidence supports the existence of two distinct pathogenetic pathways for bladder cancer development, corresponding to two distinct biological and clinical phenotypes: non-muscle-invasive and muscle-invasive urothelial carcinoma
- Disruption to the PI3K–AKT–mTOR pathway and alterations in the tyrosine kinase receptor gene *FGFR3* and the oncogene *HRAS* are associated with non-muscle-invasive bladder cancer
- The main genetic alterations underlying muscle-invasive bladder cancer involve tumor suppressor genes encoding proteins that regulate cell cycle and apoptosis pathways, including *TP53*, *CDKN2A*, *CCND1*, *CDKN1B*, and *RB1*
- Delineation of the molecular pathways involved in bladder oncogenesis has fueled the quest for much-needed prognostic biomarkers, new targeted therapies, and markers of therapy response
- A 'molecular grade' (based on *FGFR3* and *MIB1* expression) and a multitarget fluorescence *in situ* hybridization (FISH) assay have both achieved sufficient validation to be included in prospective multi-institutional trials
- A panel of multiple cell cycle control markers—*TP53*, *CDKN1B*, *CDKN1A*, *CDKN2A*, *CCND1*, and *RBL1*—offers superior prognostic power over single marker approaches, and merits further rigorous prospective validation

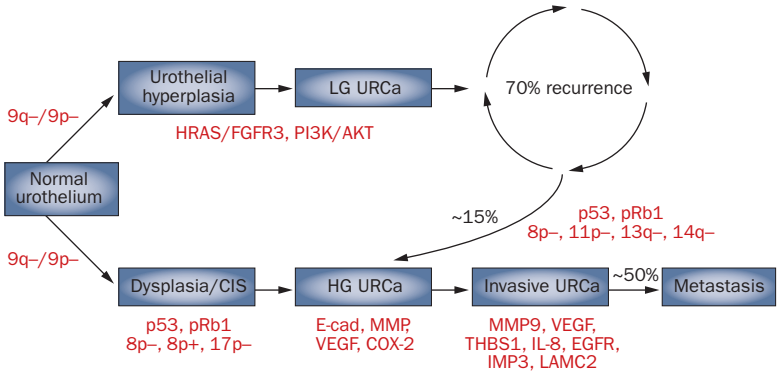

Figure 1 | The divergent molecular pathways of oncogenesis in non-muscle-invasive and muscle-invasive bladder cancers. Genetic alterations are depicted for key stages of disease progression. The main alterations underlying noninvasive low-grade urothelial carcinoma development involve *FGFR3*, *HRAS* and mTOR pathway member genes. By contrast, alterations leading to progression to noninvasive high-grade and muscle-invasive urothelial carcinoma involve the tumor suppressor genes *TP53* and *RBL1*, as well as several tumor microenvironment alterations. Abbreviations: AKT, v-akt murine thymoma viral oncogene; CIS, carcinoma *in situ*; COX-2, cyclooxygenase 2; E-cad, cadherin 1 (E-cadherin); EGFR, epidermal growth factor receptor; FGFR3, fibroblast growth factor receptor 3; HG URCa, noninvasive high-grade urothelial carcinoma; HRAS, v-Ha-ras Harvey rat sarcoma viral oncogene homolog; IL-8, interleukin 8; LAMC2, laminin subunit gamma-2; LG URCa, noninvasive low-grade urothelial carcinoma; MMP, matrix metalloproteinase; p53, tumor protein p53; PI3K, phosphoinositide-3-kinase; pRb1, retinoblastoma-like protein 1; THBS1, thrombospondin 1; URCa, urothelial carcinoma of urinary bladder; VEGF, vascular endothelial growth factor.

gained approval for screening high-risk patients with hematuria and a history of smoking or smoking exposure. With the exception of one study,[29] the multitarget (multicolor) FISH urine assay has been shown to be more sensitive than routine cytology, with reported sensitivity and specificity ranges of 69–87% and 89–96%, respectively.[28,30,31] The assay also seems to enhance the sensitivity of routine urine cytology analysis and can be used in combination with routine cytology as a reflex test in patients with atypical cytology.[28,30,31]

An additional advantage of urine-based FISH testing could be the "anticipatory positive" category of patients identified by this assay, which can detect urinary molecular alterations caused by bladder cancer several months before cancer can be detected by routine cytology or cystoscopy. In a study by Yoder and colleagues,[31] two-thirds of patients categorized as "anticipatory positive" (accounting for 27% of the total number of patients) developed bladder cancer that was subsequently detected by cystoscopy up to 29 months later. Such encouraging results indicate great potential for FISH assays in the early detection of bladder cancer and in the allocation of rigorous, frequent follow-up cystoscopy appointments to patients who are at increased risk of progression.[32–35]

In addition, several studies have pointed to a potential prognostic role of multitarget FISH analysis.[26,27,36–38] Maffezini *et al.*[37] were able to demonstrate that low-risk FISH-positive patients, with 9p21 loss or chromosome 3 abnormalities, experienced a higher rate of recurrence than patients with negative FISH results. The recurrence rate was even greater in high-risk FISH-positive patients with abnormalities in chromosome 7 or 17. Kawauchi *et al.*[26] (using bladder washings) and Kruger *et al.*[27] (using formalin-fixed paraffin-embedded [FFPE] transurethral biopsy samples) independently showed that loss of 9p21 predicted recurrence, but not progression, in patients with NMIBC. Furthermore, both Savic *et al.*[36] and Whitson *et al.*[38] demonstrated that urine cytology and FISH analysis on post-BCG bladder washings are predictive of BCG therapy failure in patients with NMIBC. This promising prognostic role for multitarget FISH awaits a prospective randomized trial before it can be integrated into clinical practice algorithms. Clear guidelines for interpreting and testing performance parameters, particularly in terms of interobserver reproducibility, are also needed.[39]

Receptor tyrosine kinases and PCR-based assays

A significant proportion of NMIBCs, particularly pTa and pT1 tumors, recur following transurethral resection of the bladder (TURB). A minority of cases will progress to high-grade carcinoma and, ultimately, muscle-invasive disease. Genetic instability is pivotal to this progression, and three primary genetic alterations that have been consistently associated with the pathogenesis pathway of NMIBC affect the receptor tyrosine kinase (RTK) genes *FGFR3*, *HRAS*, and *PIK3CA*.[3,40,41] Studies have indicated the potential prognostic value of evaluating expression levels of RTK genes such as *FGFR3*, *EGFR*, *ERBB2*, and *ERBB3* in patients with NMIBC and muscle-invasive bladder cancer (MIBC).[4,13,42–50,61] *FGFR3* mutations are a particularly common occurrence in patients with NMIBC and *FGFR3* expression levels could theoretically be used alone, or in combination with *HRAS* and *PIK3CA* expression status, as markers of early recurrence during surveillance. Both Zuiverloon *et al.*[51] and Miyake *et al.*[52] independently developed sensitive PCR-based assays for detecting *FGFR3* mutations in voided urine. A positive urine sample, as determined by the assay developed by Zuiverloon's group, was associated with concomitant or future recurrence in 81% of NMIBC patients. An even higher positive predictive value of 90% was achieved in

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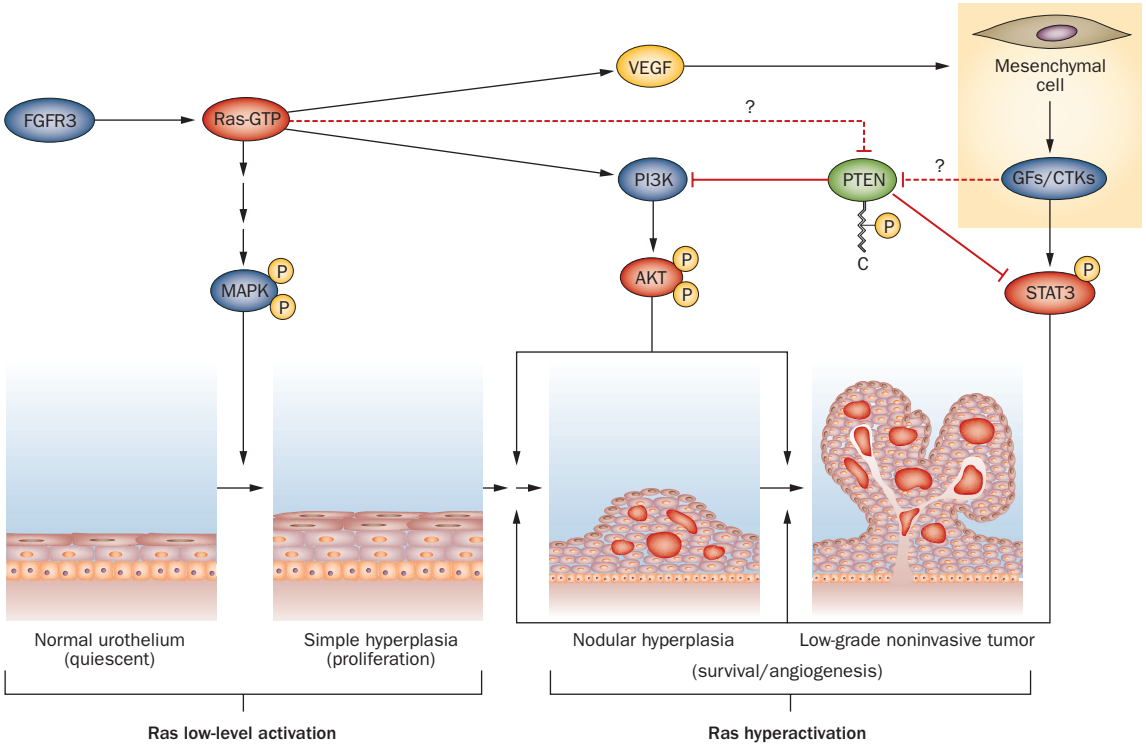

Figure 2 | Possible interactions between Ras–MAPK, JAK–STAT, and PI3K–AKT–mTOR signaling pathways associated with early oncogenesis in noninvasive papillary urothelial neoplasms. Ras activation promotes survival and angiogenesis by upregulating PI3K–AKT and JAK–STAT pathways. Inactivation of PTEN can also lead to upregulation of *AKT* and JAK–STAT pathway activation. Alternatively, *FGFR3* mutations can trigger Ras activation, leading to downstream signal transduction. Abbreviations: AKT, v-akt murine thymoma viral oncogene; CTK, cytokine; FGFR3, fibroblast growth factor receptor 3; GF, growth factor; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide-3-kinase; PTEN, phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase; Ras-GTP, constitutively active ras-GTPase; STAT3, signal transducer and activator of transcription 3 (acute-phase response factor); VEGF, vascular endothelial growth factor. Permission obtained from the American Society for Clinical Investigation © Mo, L. *et al. J. Clin. Invest.* **117**, 314–325 (2007).

patients with consecutive *FGFR3*-positive urine samples. Similarly, Miyake *et al.*[52] were able to detect *FGFR3* mutations in 53% of their 45 patients and found their assay to be superior to cytology (78% versus 0%) for detecting post-TURB recurrence in patients harboring *FGFR3* mutations in primary NMIBC tumors.

Kompier *et al.*[41] have developed a multiplex PCR assay for performing analysis of mutational 'hot spots' within *HRAS*, *KRAS*, *NRAS*, *FGFR3*, and *PIK3CA* in FFPE TURB samples. They found evidence of a mutation in at least one of these sites in up to 88% of low-grade NMIBC samples. Hernandez *et al.*[53] observed that *FGFR3* mutations were more common among neoplasms with low malignant potential (present in 77% of samples), TaG1 tumors (61%), and TaG2 tumors (58%) than among TaG3 (34%) and T1G3 tumors (17%). Multivariate analysis showed that these mutations were associated with an increased risk of recurrence in patients with NMIBC.

In addition, several studies have suggested a negative prognostic role for *ERBB2* amplification or overexpression in MIBC.[54–57] Using multivariate analysis, Bolenz *et al.*[50] found that patients harboring tumors with *ERBB2* overexpression were twice as likely to experience recurrence, and to die from their cancer, than patients with *ERBB2*-negative tumors.

Cell cycle regulators

The pathogenesis pathway for MIBC primarily involves alterations in the tumor suppressor genes responsible for cell cycle control, including *TP53*, *P16*, and *RB1*.[1,4,8] Progression of NMIBC to higher-grade muscle-invasive disease is also due to alterations in *TP53* and *RB1* (Figures 1 and 3). Early studies by Sarkis *et al.*[58–60] found *TP53* alterations to be strong independent predictors of disease progression in patients with NMIBC, MIBC, and CIS. Recent studies have supported these findings by showing an independent role of *TP53* alteration in predicting disease-free survival (DFS) and disease-specific survival (DSS) in patients with pT1 and pT2 tumors who have undergone cystectomy.[61]

Other cell cycle regulators, such as cyclin D3, cyclin D1, cyclin-dependent kinase inhibitor 2A (also known as p16), CDK-interacting protein 1 (also known as p21), and cyclin-dependent kinase inhibitor 1B (also known as p27) have also been evaluated as prognosticators for NMIBC.[24,62–67] Lopez-Beltran *et al.*[62,63] identified roles for the overexpression of cyclin D3 and cyclin D1 in predicting progression in patients with pTa and pT1 tumors. However, their findings were contradicted by a subsequent report from Shariat and colleagues,[24] emphasizing the need for further validation in large multi-institutional patient cohorts.

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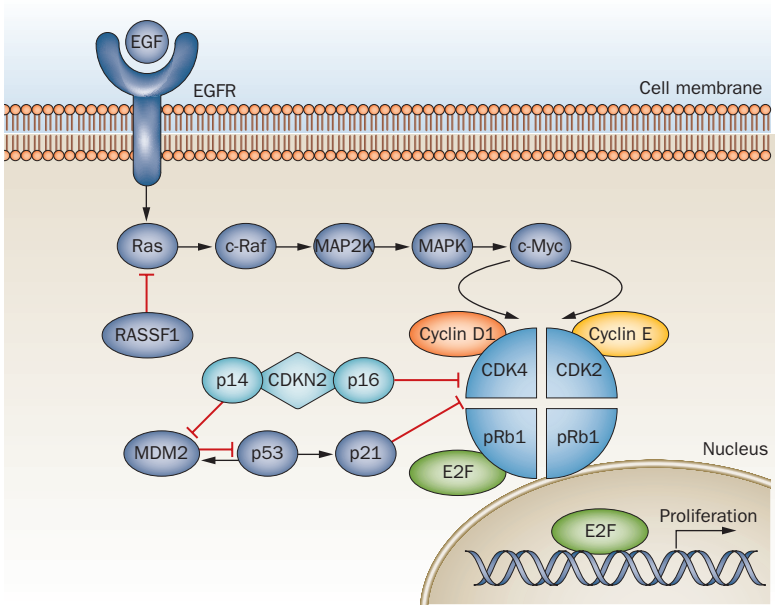

Figure 3 | Receptor tyrosine kinase (EGFR, Ras, MAP2K, and MAPK) and cell cycle regulator (p14, p16, p53, p21, cyclin D1, cyclin E, and pRb1) pathways in urothelial carcinoma. Black arrows and red lines represent stimulation and inhibition, respectively. Abbreviations: CDK2, cyclin-dependent kinase 2; CDK4, cyclin-dependent kinase 4; CDKN2, cyclin-dependent kinase inhibitor 2A; c-Myc, Myc proto-oncogene protein; c-Raf, v-raf-1 murine leukemia viral oncogene homolog 1; E2F, E2F transcription factor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; MAP2K, mitogen-activated protein kinase kinase; MAPK, mitogen-activated protein kinase; MDM2, DNA replication licensing factor MCM2; p14, cyclin-dependent kinase 2 associated protein 2; p16, cyclin-dependent kinase inhibitor 2A; p21, CDK-interacting protein 1; p53, tumor protein p53; pRb1, retinoblastoma-like protein 1; Ras, v-Ha-ras Harvey rat sarcoma viral oncogene homolog; RASSF1, Ras association (RalGDS/AF-6) domain family member 1.

Prognostic tests that assess the expression levels of multiple cell cycle regulators are emerging for the management of both NMIBC and MIBC.[16,18,68–70] Shariat *et al.*[71] found that patients with NMIBC and alterations in *TP53*, *CDKN1A*, *RBL1*, and *CDKN1B* had a significantly reduced DFS compared with patients with alterations in only three of these markers. This trend continued and the negative predictive effect decreased with decreasing number of altered markers. The same group later found that combining *TP53*, *CDKN1B*, and *MKI67* expression assessment on pT1 radical cystectomy specimens further improved the accuracy of DFS and DSS predictions.[70] Similarly, Chatterjee *et al.*[16] demonstrated synergism in the prognostic power of assessing multiple molecular markers—*TP53*, *RBL1*, and *CDKN1A*—in patients undergoing cystectomy for MIBC. The superiority of a multimarker approach over the current single-marker approaches certainly merits further assessment.[58–60,70–72,85] Assuming that additional large prospective trials confirm these promising findings, a multimarker approach could soon be integrated into the standard of care for bladder cancer management.

Hypermethylation and MSP-PCR

Epigenetic analysis is gaining momentum as a non-invasive diagnostic tool for the screening and surveillance of patients with bladder cancer, and has also shown potential as a prognostic tool.[73–86] In an early study by Catto and colleagues,[81] 11 CpG promoter islands were analyzed for hypermethylation using methylation-specific PCR (MSP-PCR) on samples from 116 bladder tumors and 164 upper urinary tract tumors. Promoter methylation was found in 86% of all tumors, was noticeably more common in upper tract tumors than in bladder cancer samples, and was generally associated with advanced tumor stage and higher rates of tumor progression and mortality. Importantly, multivariate analysis of the data established an association between methylation at the *RASSF1A* and *DAPK1* gene promoters and disease progression that occurred independently of tumor stage and grade.

Following on from this study, the same group[86] used quantitative MSP-PCR on 17 candidate gene promoters to identify five genes associated with disease progression: *RASSF1A*, *CDH1*, *TNFRSF25*, *EDNRB*, and *APC*. Multivariate analysis revealed that the overall degree of methylation was more significantly associated with subsequent progression and mortality than with tumor stage. An epigenetic predictive model, developed using artificial intelligence techniques, was able to predict the likelihood and timing of progression with 97% specificity and 75% sensitivity. The authors developed a neuro-fuzzy model to examine the importance of methylation at the various gene promoters. Two modeling strategies were used to rank the extent of each individual promoter's influence upon the predictive accuracy of the model: a "leave-one-out" approach removed each variable in turn from the model, and a "selectivity" approach spanned each variable from minimum to maximum while fixing all other variables within the model to nominal values. By comparing this ranking to the methylation profile of the tumor, the neuro-fuzzy model was able to predict the presence and timing of disease progression.

In another study of the diagnostic role of promoter hypermethylation, Lin *et al.*[74] used MSP-PCR to determine the methylation status of 4 genes—*CDH1*, *CDKN2A*, *CDK2AP2*, and RASSF1A—in DNA taken from the primary tumors and urine sediment of 57 individuals with bladder cancer. Hypermethylation analysis *of CDH1*, *CDK2AP2*, or *RASSF1A* in urine sediment DNA detected 85% of superficial and low-grade bladder tumors, 79% of high-grade tumors, and 75% of invasive bladder cancers. The study highlighted the great potential of this test in detecting NMIBC. A similar diagnostic role for methylation status was found by Cabello *et al.*[76] using a novel technology—methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)—to analyze the methylation status of 25 tumor-suppressor genes thought to have a role in bladder cancer oncogenesis, including *PTEN*, *CD44*, *WT1*, *GSTP1*, *BRCA1*, *BRCA2*, *RBL1*, *TP53*, *TP73*, *RARB*, *VHL*, *ESR1*, *PAX5*, *CDKN2A*, and *PAX6*. The authors found that *BRCA1*, *WT1*, and *RARB* were the most frequently methylated sites, with receiver operating characteristic (ROC) curve analyses revealing significant diagnostic accuracy. In addition to potential roles in diagnosing and

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prognosing bladder cancer, promoter hypermethylation is providing insights into the process underlying bladder cancer oncogenesis. Research led by Dudziec[78] has shown an association between the hypermethylation of CpG islands and 'shores' around specific mirtrons and microRNAs, and bladder cancer phenotype.

Genetic profiling and array analysis

Several gene expression studies have identified sets of differentially expressed genes with potential roles in diagnosing bladder cancer and predicting recurrence and progression.[1,7–9,21,23,80,87–91] A landmark study by Sanchez-Carbayo *et al.*[7] used oligonucleotide arrays to analyze the transcription profiles of 105 patients with bladder cancer. Hierarchical clustering and supervised algorithms were used to stratify bladder tumors based on stage, nodal metastases, and overall survival. Predictive algorithms based on levels of differentially expressed proteins in both non-muscle-invasive and invasive tumors were found to be 89% accurate for tumor staging. In terms of predicting overall survival, accuracies of 82% (for the entire cohort) and 90% (for MIBC patients only) were reported. A genetic profile, built using 174 probes, was able to identify patients with positive lymph node status and poor survival. Furthermore, two independent Global Test runs—which measure the influence of a group of genes associated with lymph node metastases on overall survival status—confirmed a robust association between genetic profile, lymph node metastases, and overall survival. Synuclein, which encodes the ligand of the cannabinoid or synuclein receptor, was selected as one of the top-ranking genes for predicting lymph node invasion and overall survival. Immunohistochemical analyses of tissue arrays confirmed a significant association between synuclein expression, tumor staging, and clinical outcomes that occurred independently of clinicopathologic parameters.

Birkhahn *et al.*[21] attempted to identify genes that are predictive of recurrence and progression in patients with pTa bladder tumors. They adopted a quantitative pathway-specific approach by checking the expression of 24 key genes involved in pathways relevant to NMIBC oncogenesis (such as cell cycle, apoptosis, and angiogenesis) using real-time PCR on tumor biopsy samples taken at initial patient presentation. They showed that *CCND3* expression is highly sensitive and moderately specific for predicting recurrence (97% and 63%, respectively). *HRAS*, *E2F1*, *BIRC5*, and *KDR* expression levels were all independently predictive of progression, and multivariate analysis of combined *HRAS*, *VEGFR2*, and *VEGFA* expression status predicted progression with an impressive 81% sensitivity and 94% specificity.

Mengual *et al.*[23] performed gene expression analysis on 341 urine samples from patients with NMIBC and MIBC and 235 controls using TaqMan® arrays (Applied Biosystems, Carlsbad, CA, USA). A panel of 12 differentially expressed genes—*ANXA10*, *AHNAK2*, *CTSE*, *CRH*, *IGF2*, *KLF9*, *KRT20*, *MAGEA3*, *POSTN*, *PPP1R14D*, *SLC1A6*, and *TERT*—obtained from comparing the gene expression signatures of urine samples from patients with and without urothelial carcinoma demonstrated very high sensitivity and specificity for identifying bladder cancer (98% and 99%, respectively). The addition of two other genes that are differentially expressed between NMIBC and MIBC (*ASAM* and *MCM10*) enabled accurate prediction of tumor aggressiveness (79% sensitivity and 92% specificity). This signature was validated in voided urine samples and shown to maintain high levels of accuracy.

In an integrated genetic–epigenetic approach, Serizawa *et al.*[80] screened for mutations in 6 genes (*FGFR3*, *PIK3CA*, *TP53*, *HRAS*, *NRAS*, and *KRAS*) and quantitatively assessed the promoter methylation status of 11 additional genes (*APC*, *ARF*, *DBC1*, *INK4A*, *RARB*, *RASSF1A*, *SFRP1*, *SFRP2*, *SFRP4*, *SFRP5*, and *WIF1*) in NMIBC tumor biopsies and corresponding urine samples from 118 patients and 33 controls. A total of 95 oncogenic mutations and 189 hypermethylation events were detected. Assessing *FGFR3* mutations in combination with the methylation status of three markers (*APC*, *RASSF1A*, and *SFRP2*) provided sensitivities of 90% in tumors and 62% in urine, with 100% specificity for both. Evaluation of the complete panel of markers provided sensitivities of 93% and 70% in biopsy and urine samples, respectively.

Selecting patients for neoadjuvant chemotherapy on the basis of their risk of node-positive disease could benefit high-risk patients and spare low-risk patients from the associated toxic effects and delay to cystectomy. Smith *et al.*[92] recently reported a 20-gene expression model that is able to predict pathological nodal status independently of standard clinicopathologic prognostic criteria. They tested this model using primary tumor tissue samples from three independent cohorts of patients with no clinical evidence of nodal metastasis; initially in two separate cohorts of 90 and 66 patients and then in a phase III trial involving 185 patients.

Molecular grading and signatures

Van Rhijn *et al.*[93] proposed a molecular grade parameter, based on a combination of *FGFR3* gene mutation status and MIB1 labeling index, as an alternative to pathologic grading for NMIBC. The same group recently validated this parameter[42] using the European Organization for Research and Treatment of Cancer (EORTC) NMIBC risk calculator,[94] which generates a weighted score from six variables (WHO 1973 grade, stage, presence of CIS, multiplicity, size, and prior recurrence rate). The molecular grade was found to be more reproducible than the pathologic grade (89% versus 41–74% reproducibility). *FGFR3* mutations correlated with favorable disease parameters, whereas increased *MIB1* expression was frequently associated with pT1 tumors, high-grade disease, and high EORTC risk scores. Molecular grade held independent significance for predicting progression and DSS, and the addition of molecular grade to the EORTC model for progression increased the predictive accuracy from 74.9% to 81.7%.[93]

In another study, Lindgren *et al.*[95] suggested that a combined molecular and histopathological classification

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of bladder cancer might prove more powerful in predicting outcomes and stratifying treatment. The authors combined gene expression analysis, whole genome array comparative genomic hybridization (CGH) analysis, and mutational analysis of *FGFR3*, *PIK3CA*, *KRAS*, *HRAS*, *NRAS*, *TP53*, *CDKN2A*, and *TSC1* to identify two intrinsic molecular signatures (MS1 and MS2). Genomic instability was the most distinguishing genomic feature of the MS2 signature, which occurred independently of *TP53* and *MDM2* alterations. Genetic signatures were validated by two independent data sets that successfully classified urothelial carcinomas into low-grade or high-grade NMIBC and MIBC tumors with high precision and sensitivity. Furthermore, a gene expression signature that independently predicts metastasis and DFS was also defined. These data clearly support the role of molecular grading as a complement to standard pathologic grading.

Tumor proliferation indices

Tumor proliferation index, calculated from either *KI67* or *MIB1* expression levels, has consistently been shown to be an effective prognosticator for bladder cancer.[42,63,66,93,96–99] MIB1 tumor proliferation index has a role in determining the prognosis of NMIBC as a component of the molecular grade parameter proposed by Van Rihjn *et al.*[93] An independent prognostic role for KI67 tumor proliferation index has also been shown, with KI67 indices calculated from NMIBC TURB biopsy samples predicting progression-free survival (PFS) and DSS.[96]

A prognostic role for tumor proliferation index has also been established for MIBC. The Bladder Consortium Multi-Institutional Trial (which included 7 institutions and 713 patients) was able to confirm a prognostic role for proliferation index using cystectomy specimens,[98] building upon the initial findings of Margulis *et al.*[97] In later work, the KI67 tumor proliferation index was used to improve predictive accuracy for both PFS and DSS when added to standard prediction models, supporting a role for the KI67 biomarker in the stratification of patients under consideration for perioperative systemic chemotherapy.[98]

Ploidy and morphometric analysis

Several reports have suggested an independent prognostic role for ploidy and S phase analysis in the management of NMIBC.[99–106] Ploidy analysis can be performed by flow cytometry or automated image cytometry (ICM) and can be applied to urine cytology specimens, as well as biopsy supernatant[101] and disaggregated TURB FFPE specimens.[102] In one of the largest studies assessing DNA ploidy in NMIBC tumors (377 in the test set and 156 in the validation set), Ali-Et-Dein *et al.*[100] found that stage, DNA ploidy, tumor multiplicity, history of recurrence, tumor configuration, and type of adjuvant therapy can all independently predict recurrence. Recurrence (3 months after initial treatment), grade, and DNA ploidy were also effective predictors of progression to muscle-invasive disease. Baak *et al.*[102] supported these findings by showing that ploidy status and S phase kinetics measured by ICM are strong independent predictors of recurrence and progression in patients with pTa and pT1 tumors. Although the data collected so far are encouraging, ploidy analysis still awaits large prospective randomized trials before ICM or flow cytometry techniques can be introduced into management algorithms for patients with NMIBC.

Other emerging potential biomarkers

Another genre of biomarkers with encouraging, but less robust, data regarding their potential prognostic power in bladder cancer management are tumor microenvironment markers, including the cell adhesion markers E-cadherin and N-cadherin[12,107] and angiogenesis modulators such as the hypoxia-inducible factors HIF1A and HIF2, vascular endothelial growth factor (VEGF), carbonic anhydrase IX protein (CAIX), and thrombospondin-1.[10,11,108–112] mTOR pathway markers have also been shown to have a potential prognostic role in bladder cancer,[12,107,113–115] and the mitotic serine-threonine kinase Aurora-A—which has an integral role in cell proliferation—has also been investigated in this setting.[116,117] Finally, microRNA profile alterations have shown promise within this field and are likely to be heavily investigated in the future as noninvasive markers for diagnosing and prognosing patients with bladder cancer.[78,118–120]

Bladder cancer biomarkers in targeted therapy

Key players in the Ras–MAPK and PI3K–AKT–mTOR pathways, as well as the angiogenesis pathway of the tumor microenvironment, offer promising opportunities for new targeted treatments of bladder cancer.[17,41,113,115,121,122,131–134] The RTKs, in particular, have been a focus for investigation. In a multicenter phase II trial, 44 patients with advanced metastatic bladder cancer testing positive for ERBB2 (also known as HER2) were treated with a combination of carboplatin, paclitaxel, gemcitabine, and trastuzumab (a humanized monoclonal antibody against ERBB2).[135] Approximately 70% of treated patients demonstrated a partial (59%) or complete (11%) response, with a median overall survival of 14.1 months. Improved response rates were observed in patients with 3+ ERBB2 expression scores and *ERBB2* gene amplification (determined by IHC and FISH respectively) than in patients with 2+ ERBB2 expression scores and FISH-negative tumors.[135] Interestingly, in contrast to the strong correlation between *ERBB2* gene amplification and 3+ ERBB2 IHC expression scores typically observed in breast cancer patients, ERBB2 overexpression was not commonly associated with *ERBB2* gene amplification in bladder cancer.[136]

An ongoing randomized phase II trial has been set up to evaluate the role of cetuximab, an anti-EGFR recombinant humanized murine monoclonal antibody.[121] Patients with metastatic, locally recurrent, or nonresectable disease are being treated with standard gemcitabine and carboplatin (GC) chemotherapy with or without cetuximab. By blocking EGF binding to the extracellular EGFR domain, cetuximab inhibits the downstream signal transduction pathway, accounting for

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its antiproliferative activity in solid tumors. In bladder cancer cells, an additional antiangiogenic effect could also at play.[137,138] However, the phase II Cancer and Leukemia Group B trial, which investigated the role of gefitinib (a small molecule inhibitor of *EGFR*) in patients with advanced bladder cancer found no survival or time to progression advantage for combination therapy over GC therapy alone.[139,140]

Lapatinib—a tyrosine kinase inhibitor (TKI) that targets both EGFR and ERBB2—is currently used in combination therapy for ERBB2-positive breast cancer.[141] The results of a phase II single-arm trial suggest a therapeutic advantage of lapatinib in patients with EGFR-positive and ERBB2-positive bladder tumors.[141] A phase II/III randomized trial is currently underway to investigate whether lapatinib could be used as maintenance therapy in patients who demonstrate an objective response to first-line chemotherapy and test positive for either EGFR or ERBB2 using IHC or FISH analysis.

The role of multitarget TKIs in bladder cancer has also been investigated, with mixed results. Although phase II trials of sorafenib (an inhibitor of *RAF1*, *BRAF*, *PDGFRB*, *KDR*, and *FLT4*) have failed to show a significant objective response, sunitinib has shown promise in a recent phase II trial ($n = 77$) at Memorial Sloan Kettering Cancer Center.[121] A clinical benefit was observed in almost one-third of these patients. A subsequent randomized double-blind phase II trial is underway to investigate the efficacy of sunitinib in delaying tumor progression in patients with a positive initial response to standard chemotherapy.[142]

In an attempt to target the dependence of bladder tumors on angiogenesis, monoclonal antibodies and small-molecule inhibitors of angiogenesis are currently under investigation for patients with advanced disease. A potential role has been proposed for bevacizumab, a recombinant humanized monoclonal anti-VEGF antibody, as a first-line combination therapy with GC in patients with metastatic bladder cancer.[143] A phase II study found that two-thirds of patients demonstrated an objective response, with 14% showing complete response—albeit with significant treatment-related toxicity.[143] The CALBG phase III randomized trial is now underway to assess the effect of this combination therapy on inhibiting angiogenesis in metastatic urothelial carcinoma.[121] Another phase II trial is currently investigating the benefits of administering bevacizumab in combination with M-VAC (methotrexate, vinblastine, adriamycin, and cisplatin) chemotherapy.[144]

Given the recent evidence to suggest the importance of mTOR pathway alterations in bladder cancer, a phase II trial is underway to evaluate the potential effects of treatment with everolimus—a sirolimus-derived inhibitor of the mTOR pathway that is currently used in the treatment of renal cell cancer—in patients with advanced bladder cancer.[113–115]

Conclusions

As our understanding of the complex molecular mechanisms involved in bladder cancer development deepens, our approaches to the diagnosis and management of this disease continue to evolve. In the near future, the current clinicopathology-based prognostic approaches for predicting progression in NMIBC[88,145–147] (Box 1) will likely be supplemented by a molecular-guided approach, based on molecular biomarkers (Box 2). Given the advantages in expected cost and turnaround time associated with next-generation sequencing technology, a genomic approach to providing noninvasive diagnostic and predictive tools should be actively pursued.

Evidently, a rigorous validation process must precede the incorporation of novel molecular biomarkers into

Box 1 | Established clinicopathologic prognostic parameters in NMIBC and MIBC

Non-muscle-invasive bladder cancer (NMIBC)
- WHO/ISUP grade
- pT stage
- Presence of associated CIS/dysplasia
- Disease duration
- Time to, and frequency of, recurrences
- Multifocality
- Tumor size (>3 cm)
- Failure of prior BCG therapy
- Presence of lymphovascular invasion
- Depth of lamina propria invasion

Muscle-invasive bladder cancer (MIBC)
- pTNM staging
- Presence of lymphovascular invasion
- Resistance to neoadjuvant chemotherapy
- Divergent histology (micropapillary, osteoclast rich, undifferentiated/giant cell, plasmacytoid)

Box 2 | Emerging molecular prognostic markers in NMIBC and MIBC

Non-muscle-invasive bladder cancer (NMIBC)
- Multitarget FISH
- *FGFR3* mutation/overexpression
- *HRAS* alterations
- Molecular grade (mG)
- Proliferation index (KI67, MIB1)
- p53 inactivation/accumulation
- Downregulated expression of pRb1, p21, p27
- Overexpression of cyclin D3, cyclin D1
- *ERBB3/ERBB4* overexpression (protective)
- Loss of E-cadherin
- Multimarker immunoexpression analysis (p53, p27, KI67, pRb1, p21)
- Gene expression array panels
- DNA ploidy status and S phase kinetics
- Angiogenesis marker overexpression (*VEGF*, *HIF1A*, *THBS1*)
- Promoter hypermethylation (*RASSF1*, *DAPK*, *APC*, *CDH1*, *EDNRB*)

Muscle-invasive bladder cancer (MIBC)
- p53 inactivation/accumulation
- Alterations of pRb1 expression
- Loss of p21 expression
- Alteration of p16 expression
- Loss of E-cadherin
- *EGFR* overexpression
- *ERBB2* overexpression/amplification
- Angiogenesis marker overexpression (*VEGF*, *HIF1A*, *THBS1*)
- mTOR alterations
- Phos S6 expression (protective)
- Gene expression array panels
- Promoter hypermethylation (*RASSF1*, *CDH1*, *EDNRB*)

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clinical management. The results of initial retrospective studies will first need to be confirmed and validated in large independent cohorts, followed by well-controlled, multi-institutional randomized prospective trials. A lack of these trials has hindered the clinical utilization of several promising biomarkers to date.[35,36] For new biomarkers to be considered for integration into clinical practice, these trials should demonstrate a clear benefit over existing management algorithms.[54,148] Several targeted therapies are now under investigation that combine standard chemotherapy agents and novel biomarkers—either as first-line treatments or as maintenance therapy to prolong response in patients with advanced bladder cancer. Exciting challenges and opportunities await investigators with a special interest in bladder cancer, and clinical investigations will likely bring about much-needed progress in the management of patients with this disease.

Review criteria

A systematic review of the literature was performed to identify English language full-text manuscripts published between 1990 and 2011. Cited articles were found by searching PubMed using the terms "molecular", "bladder cancer", "prognostic", "theranostic", and "targeted therapy". Reference lists of included papers were also checked for further relevant articles. Larger studies with statistical vigor were given priority.

1. Mitra, A. P., Datar, R. H. & Cote, R. J. Molecular pathways in invasive bladder cancer: new insights into mechanisms, progression, and target identification. *J. Clin. Oncol.* **24**, 5552–5564 (2006).
2. Mitra, A. P. & Cote, R. J. Molecular screening for bladder cancer: progress and potential. *Nat. Rev. Urol.* **7**, 11–20 (2010).
3. Mitra, A. P. & Cote, R. J. Molecular pathogenesis and diagnostics of bladder cancer. *Ann. Rev. Pathol.* **4**, 251–285 (2009).
4. Wu, X. R. Urothelial tumorigenesis: a tale of divergent pathways. *Nat. Rev. Cancer* **5**, 713–725 (2005).
5. Mo, L. *et al.* Hyperactivation of Ha-ras oncogene, but not Ink4a/Arf deficiency, triggers bladder tumorigenesis. *J. Clin. Invest.* **117**, 314–325 (2007).
6. Rabbani, F. *et al.* Prognostic significance of p27(Kip1) expression in bladder cancer. *BJU Int.* **100**, 259–263 (2007).
7. Sanchez-Carbayo, M., Socci, N. D., Lozano, J., Saint, F. & Cordon-Cardo, C. Defining molecular profiles of poor outcome in patients with invasive bladder cancer using oligonucleotide microarrays. *J. Clin. Oncol.* **24**, 778–789 (2006).
8. Sanchez-Carbayo, M. *et al.* Molecular profiling of bladder cancer using cDNA microarrays: defining histogenesis and biological phenotypes. *Cancer Res.* **62**, 6973–6980 (2002).
9. Sanchez-Carbayo, M. & Cordon-Cardo, C. Applications of array technology: identification of molecular targets in bladder cancer. *Br. J. Cancer* **89**, 2172–2177 (2003).
10. Ioachim, E. *et al.* Hypoxia-inducible factors HIF-1alpha and HIF-2alpha expression in bladder cancer and their associations with other angiogenesis-related proteins. *Urol. Int.* **77**, 255–263 (2006).
11. Ioachim, E. *et al.* Thrombospondin-1 expression in urothelial carcinoma: prognostic significance and association with p53 alterations, tumour angiogenesis and extracellular matrix components. *BMC Cancer* **6**, 140 (2006).
12. Lascombe, I. *et al.* N-cadherin as a novel prognostic marker of progression in superficial urothelial tumors. *Clin. Cancer Res.* **12**, 2780–2787 (2006).
13. Rotterud, R., Nesland, J. M., Berner, A. & Fossa, S. D. Expression of the epidermal growth factor receptor family in normal and malignant urothelium. *BJU Int.* **95**, 1344–1350 (2005).
14. Highshaw, R. A., McConkey, D. J. & Dinney, C. P. Integrating basic science and clinical research in bladder cancer: update from the first bladder Specialized Program of Research Excellence (SPORE). *Curr. Opin. Urol.* **14**, 295–300 (2004).
15. Clairotte, A. *et al.* Expression of E-cadherin and alpha-, beta-, gamma-catenins in patients with bladder cancer: identification of gamma-catenin as a new prognostic marker of neoplastic progression in T1 superficial urothelial tumors. *Am. J. Clin. Pathol.* **125**, 119–126 (2006).
16. Chatterjee, S. J. *et al.* Combined effects of p53, p21, and pRb expression in the progression of bladder transitional cell carcinoma. *J. Clin. Oncol.* **22**, 1007–1013 (2004).
17. Beekman, K. W., Bradley, D. & Hussain, M. New molecular targets and novel agents in the treatment of advanced urothelial cancer. *Semin. Oncol.* **34**, 154–164 (2007).
18. Shariat, S. F., Ashfaq, R., Sagalowsky, A. I. & Lotan, Y. Predictive value of cell cycle biomarkers in nonmuscle invasive bladder transitional cell carcinoma. *J. Urol.* **177**, 481–487 (2007).
19. Miyamoto, H. *et al.* Infrequent somatic mutations of the p16 and p15 genes in human bladder cancer: p16 mutations occur only in low-grade and superficial bladder cancers. *Oncol. Res.* **7**, 327–330 (1995).
20. Miyamoto, H. *et al.* Analyses of p53 gene mutations in primary human bladder cancer. *Oncol. Res.* **5**, 245–249 (1993).
21. Birkhahn, M. *et al.* Predicting recurrence and progression of noninvasive papillary bladder cancer at initial presentation based on quantitative gene expression profiles. *Eur. Urol.* **57**, 12–20 (2010).
22. Cheng, L. *et al.* The origins of urothelial carcinoma. *Expert Rev. Anticancer Ther.* **10**, 865–880 (2010).
23. Mengual, L. *et al.* Gene expression signature in urine for diagnosing and assessing aggressiveness of bladder urothelial carcinoma. *Clin. Cancer Res.* **16**, 2624–2633 (2010).
24. Shariat, S. F., Ashfaq, R., Sagalowsky, A. I. & Lotan, Y. Association of cyclin D1 and E1 expression with disease progression and biomarkers in patients with nonmuscle-invasive urothelial cell carcinoma of the bladder. *Urol. Oncol.* **25**, 468–475 (2007).
25. Stein, J. P. *et al.* Radical cystectomy in the treatment of invasive bladder cancer: long-term results in 1,054 patients. *J. Clin. Oncol.* **19**, 666–675 (2001).
26. Kawauchi, S. *et al.* 9p21 index as estimated by dual-color fluorescence *in situ* hybridization is useful to predict urothelial carcinoma recurrence in bladder washing cytology. *Hum. Pathol.* **40**, 1783–1789 (2009).
27. Kruger, S., Mess, F., Bohle, A. & Feller, A. C. Numerical aberrations of chromosome 17 and the 9p21 locus are independent predictors of tumor recurrence in non-invasive transitional cell carcinoma of the urinary bladder. *Int. J. Oncol.* **23**, 41–48 (2003).
28. Skacel, M. *et al.* Multitarget fluorescence *in situ* hybridization assay detects transitional cell carcinoma in the majority of patients with bladder cancer and atypical or negative urine cytology. *J. Urol.* **169**, 2101–2105 (2003).
29. Moonen, P. M. *et al.* UroVysion compared with cytology and quantitative cytology in the surveillance of non-muscle-invasive bladder cancer. *Eur. Urol.* **51**, 1275–80 (2007).
30. Sarosdy, M. F. *et al.* Use of a multitarget fluorescence *in situ* hybridization assay to diagnose bladder cancer in patients with hematuria. *J. Urol.* **176**, 44–47 (2006).
31. Yoder, B. J. *et al.* Reflex UroVysion testing of bladder cancer surveillance patients with equivocal or negative urine cytology: a prospective study with focus on the natural history of anticipatory positive findings. *Am. J. Clin. Pathol.* **127**, 295–301 (2007).
32. Fritsche, H. M. *et al.* Multicolor FISH (UroVysion) facilitates follow-up of patients with high-grade urothelial carcinoma of the bladder. *Am. J. Clin. Pathol.* **134**, 597–603 (2010).
33. Karnwal, A. *et al.* The role of fluorescence *in situ* hybridization assay for surveillance of non-muscle invasive bladder cancer. *Can. J. Urol.* **17**, 5077–5081 (2010).
34. Schlomer, B. J., Ho, R., Sagalowsky, A., Ashfaq, R. & Lotan, Y. Prospective validation of the clinical usefulness of reflex fluorescence *in situ* hybridization assay in patients with atypical cytology for the detection of urothelial carcinoma of the bladder. *J. Urol.* **183**, 62–67 (2010).
35. Ferra, S. *et al.* Reflex UroVysion testing in suspicious urine cytology cases. *Cancer* **117**, 7–14 (2009).
36. Savic, S. *et al.* The prognostic value of cytology and fluorescence *in situ* hybridization in the follow-up of nonmuscle-invasive bladder cancer after intravesical bacillus Calmette-Guerin therapy. *Int. J. Cancer* **124**, 2899–2904 (2009).
37. Maffezzini, M. *et al.* Prognostic significance of fluorescent *in situ* hybridisation in the follow-up of non-muscle-invasive bladder cancer. *Anticancer Res.* **30**, 4761–4765 (2010).
38. Whitson, J., Berry, A., Carroll, P. & Konety, B. A multicolour fluorescence *in situ* hybridization test predicts recurrence in patients with high-risk superficial bladder tumours undergoing intravesical therapy. *BJU Int.* **104**, 336–339 (2009).
39. Renshaw, A. A. UroVysion, urine cytology, and the College of American Pathologists: where should we go from here? *Arch. Pathol. Lab. Med.* **134**, 1106–1107 (2010).

© 2011 Macmillan Publishers Limited. All rights reserved

40. Lopez-Knowles, E. *et al.* PIK3CA mutations are an early genetic alteration associated with FGFR3 mutations in superficial papillary bladder tumors. *Cancer Res.* **66**, 7401–7404 (2006).
41. Kompier, L. C. *et al.* FGFR3, HRAS, KRAS, NRAS and PIK3CA mutations in bladder cancer and their potential as biomarkers for surveillance and therapy. *PLoS ONE* **5**, e13821 (2010).
42. van Rhijn, B. W. *et al.* Molecular grade (FGFR3/MIB-1) and EORTC risk scores are predictive in primary non-muscle-invasive bladder cancer. *Eur. Urol.* **58**, 433–441 (2010).
43. Mason, R. A. *et al.* EGFR pathway polymorphisms and bladder cancer susceptibility and prognosis. *Carcinogenesis* **30**, 1155–1160 (2009).
44. Simonetti, S. *et al.* Role of polysomy 17 in transitional cell carcinoma of the bladder: immunohistochemical study of HER2/neu expression and fish analysis of c-erbB-2 gene and chromosome 17. *Int. J. Surg. Pathol.* **17**, 198–205 (2009).
45. Latif, Z. *et al.* HER2/neu gene amplification and protein overexpression in G3 pT2 transitional cell carcinoma of the bladder: a role for anti-HER2 therapy? *Eur. J. Cancer* **40**, 56–63 (2004).
46. Gandour-Edwards, R. *et al.* Does HER2/neu expression provide prognostic information in patients with advanced urothelial carcinoma? *Cancer* **95**, 1009–1015 (2002).
47. Eissa, S., Ali, H. S., Al Tonsi, A. H., Zaglol, A. & El Ahmady, O. HER2/neu expression in bladder cancer: relationship to cell cycle kinetics. *Clin. Biochem.* **38**, 142–148 (2005).
48. Billerey, C. *et al.* Frequent FGFR3 mutations in papillary non-invasive bladder (pTa) tumors. *Am. J. Pathol.* **158**, 1955–1959 (2001).
49. Leibl, S. *et al.* EGFR expression in urothelial carcinoma of the upper urinary tract is associated with disease progression and metaplastic morphology. *APMIS* **116**, 27–32 (2008).
50. Bolenz, C. *et al.* Human epidermal growth factor receptor 2 expression status provides independent prognostic information in patients with urothelial carcinoma of the urinary bladder. *BJU Int.* **106**, 1216–1222 (2010).
51. Zuiverloon, T. C. *et al.* Fibroblast growth factor receptor 3 mutation analysis on voided urine for surveillance of patients with low-grade non-muscle-invasive bladder cancer. *Clin. Cancer Res.* **16**, 3011–3018 (2010).
52. Miyake, M. *et al.* Fibroblast growth factor receptor 3 mutation in voided urine is a useful diagnostic marker and significant indicator of tumor recurrence in non-muscle invasive bladder cancer. *Cancer Sci.* **101**, 250–258 (2010).
53. Hernandez, S. *et al.* Prospective study of FGFR3 mutations as a prognostic factor in nonmuscle invasive urothelial bladder carcinomas. *J. Clin. Oncol.* **24**, 3664–3671 (2006).
54. Bolenz, C. & Lotan, Y. Translational research in bladder cancer: from molecular pathogenesis to useful tissue biomarkers. *Cancer Biol. Ther.* **10**, 407–415 (2010).
55. Chakravarti, A. *et al.* Expression of the epidermal growth factor receptor and Her-2 are predictors of favorable outcome and reduced complete response rates, respectively, in patients with muscle-invading bladder cancers treated by concurrent radiation and cisplatin-based chemotherapy: a report from the Radiation Therapy Oncology Group. *Int. J. Radiat. Oncol. Biol. Phys.* **62**, 309–317 (2005).
56. Jimenez, R. E. *et al.* Her-2/neu overexpression in muscle-invasive urothelial carcinoma of the bladder: prognostic significance and comparative analysis in primary and metastatic tumors. *Clin. Cancer Res.* **7**, 2440–2447 (2001).
57. Ravery, V. *et al.* Evaluation of epidermal growth factor receptor, transforming growth factor alpha, epidermal growth factor and c-erbB2 in the progression of invasive bladder cancer. *Urol. Res.* **25**, 9–17 (1997).
58. Sarkis, A. S. *et al.* Nuclear overexpression of p53 protein in transitional cell bladder carcinoma: a marker for disease progression. *J. Natl Cancer Inst.* **85**, 53–59 (1993).
59. Sarkis, A. S. *et al.* Association of P53 nuclear overexpression and tumor progression in carcinoma *in situ* of the bladder. *J. Urol.* **152**, 388–392 (1994).
60. Sarkis, A. S. *et al.* Prognostic value of p53 nuclear overexpression in patients with invasive bladder cancer treated with neoadjuvant MVAC. *J. Clin. Oncol.* **13**, 1384–1390 (1995).
61. Shariat, S. F. *et al.* p53 expression in patients with advanced urothelial cancer of the urinary bladder. *BJU Int.* **105**, 489–495 (2010).
62. Lopez-Beltran, A. *et al.* Cyclin D3 expression in primary Ta/T1 bladder cancer. *J. Pathol.* **209**, 106–113 (2006).
63. Lopez-Beltran, A. *et al.* Prognostic factors in stage T1 grade 3 bladder cancer survival: the role of G1-S. modulators (p53, p21Waf1, p27kip1, Cyclin D1, and Cyclin D3) and proliferation index (ki67-MIB1). *Eur. Urol.* **45**, 606–612 (2004).
64. Fu, T. Y., Tu, M. S., Tseng, H. H. & Wang, J. S. Overexpression of p27kip1 in urinary bladder urothelial carcinoma. *Int. J. Urol.* **14**, 1084–1087 (2007).
65. Yin, M., Bastacky, S., Parwani, A. V., McHale, T. & Dhir, R. P16ink4 Immunoreactivity is a reliable marker for urothelial carcinoma *in situ*. *Hum. Pathol.* **39**, 527–535 (2008).
66. Kruger, S., Mahnken, A., Kausch, I. & Feller, A. C. P16 immunoreactivity is an independent predictor of tumor progression in minimally invasive urothelial bladder carcinoma. *Eur. Urol.* **47**, 463–467 (2005).
67. Sgambato, A. *et al.* Loss of P27Kip1 expression correlates with tumor grade and with reduced disease-free survival in primary superficial bladder cancers. *Cancer Res.* **59**, 3245–3250 (1999).
68. Garcia del Muro, X. *et al.* p53 and p21 Expression levels predict organ preservation and survival in invasive bladder carcinoma treated with a combined-modality approach. *Cancer* **100**, 1859–1867 (2004).
69. Shariat, S. F. *et al.* Multiple biomarkers improve prediction of bladder cancer recurrence and mortality in patients undergoing cystectomy. *Cancer* **112**, 315–325 (2008).
70. Shariat, S. F. *et al.* Predictive value of combined immunohistochemical markers in patients with pT1 urothelial carcinoma at radical cystectomy. *J. Urol.* **182**, 78–84 (2009).
71. Shariat, S. F., Ashfaq, R., Sagalowsky, A. I. & Lotan, Y. Predictive value of cell cycle biomarkers in nonmuscle invasive bladder transitional cell carcinoma. *J. Urol.* **177**, 481–487 (2007).
72. Dalbagni, G. *et al.* Molecular genetic alterations of chromosome 17 and p53 nuclear overexpression in human bladder cancer. *Diagn. Mol. Pathol.* **2**, 4–13 (1993).
73. Nishiyama, N. *et al.* Genome-wide DNA methylation profiles in urothelial carcinomas and urothelia at the precancerous stage. *Cancer Sci.* **101**, 231–240 (2010).
74. Lin, H. H. *et al.* Increase sensitivity in detecting superficial, low grade bladder cancer by combination analysis of hypermethylation of E-cadherin, p16, p14, RASSF1A genes in urine. *Urol. Oncol.* **28**, 597–602 (2010).
75. Vinci, S. *et al.* Quantitative methylation analysis of BCL2, hTERT, and DAPK promoters in urine sediment for the detection of non-muscle-invasive urothelial carcinoma of the bladder: a prospective, two-center validation study. *Urol. Oncol.* **29**, 150–156 (2009).
76. Cabello, M. J. *et al.* Multiplexed methylation profiles of tumor suppressor genes in bladder cancer. *J. Mol. Diagn.* **13**, 29–40 (2011).
77. Vallot, C. *et al.* A novel epigenetic phenotype associated with the most aggressive pathway of bladder tumor progression. *J. Natl Cancer Inst.* **103**, 47–60 (2011).
78. Dudziec, E. *et al.* Hypermethylation of CpG islands and shores around specific microRNAs and mirtrons is associated with the phenotype and presence of bladder cancer. *Clin. Cancer Res.* **17**, 1287–1296 (2011).
79. Wiklund, E. D. *et al.* Coordinated epigenetic repression of the miR-200 family and miR-205 in invasive bladder cancer. *Int. J. Cancer* **128**, 1327–1334 (2011).
80. Serizawa, R. R. *et al.* Integrated genetic and epigenetic analysis of bladder cancer reveals an additive diagnostic value of FGFR3 mutations and hypermethylation events. *Int. J. Cancer* **129**, 78–87 (2011).
81. Catto, J. W. *et al.* Promoter hypermethylation is associated with tumor location, stage, and subsequent progression in transitional cell carcinoma. *J. Clin. Oncol.* **23**, 2903–2910 (2005).
82. Friedrich, M. G. *et al.* Detection of methylated apoptosis-associated genes in urine sediments of bladder cancer patients. *Clin. Cancer Res.* **10**, 7457–7465 (2004).
83. Chan, M. W. *et al.* Hypermethylation of multiple genes in tumor tissues and voided urine in urinary bladder cancer patients. *Clin. Cancer Res.* **8**, 464–470 (2002).
84. Yates, D. R. *et al.* Methylational urinalysis: a prospective study of bladder cancer patients and age stratified benign controls. *Oncogene* **25**, 1984–1988 (2006).
85. Hoque, M. O. *et al.* Quantitation of promoter methylation of multiple genes in urine DNA and bladder cancer detection. *J. Natl Cancer Inst.* **98**, 996–1004 (2006).
86. Yates, D. R. *et al.* Promoter hypermethylation identifies progression risk in bladder cancer. *Clin. Cancer Res.* **13**, 2046–2053 (2007).
87. Cheng, L. *et al.* Bladder cancer: translating molecular genetic insights into clinical practice. *Hum. Pathol.* **42**, 455–481 (2011).
88. Miyamoto, H. *et al.* Low-grade papillary urothelial carcinoma of the urinary bladder: a clinicopathologic analysis of a post-World Health Organization/International Society of Urological Pathology classification cohort from a single academic center. *Arch. Pathol. Lab. Med.* **134**, 1160–1163 (2010).
89. Mitra, A. P. *et al.* Generation of a concise gene panel for outcome prediction in urinary bladder cancer. *J. Clin. Oncol.* **27**, 3929–3937 (2009).
90. Rothman, N. *et al.* A multi-stage genome-wide association study of bladder cancer identifies multiple susceptibility loci. *Nat. Genet.* **42**, 978–984 (2010).
91. Sanchez-Carbayo, M. & Cordon-Cardo, C. Molecular alterations associated with bladder cancer progression. *Semin. Oncol.* **34**, 75–84 (2007).
92. Smith, S. C. *et al.* A 20-gene model for molecular nodal staging of bladder cancer: development and prospective assessment. *Lancet Oncol.* **12**, 137–143 (2011).
93. van Rhijn, B. W. *et al.* Molecular grading of urothelial cell carcinoma with fibroblast growth

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factor receptor 3 and MIB-1 is superior to pathologic grade for the prediction of clinical outcome. *J. Clin. Oncol.* **21**, 1912–1921 (2003).
94. Sylvester, R. J. *et al.* Predicting recurrence and progression in individual patients with stage Ta T1 bladder cancer using EORTC risk tables: a combined analysis of 2596 patients from seven EORTC trials. *Eur. Urol.* **49**, 466–477 (2006).
95. Lindgren, D. *et al.* Combined gene expression and genomic profiling define two intrinsic molecular subtypes of urothelial carcinoma and gene signatures for molecular grading and outcome. *Cancer Res.* **70**, 3463–3472 (2010).
96. Quintero, A. *et al.* Ki-67 MIB1 labelling index and the prognosis of primary TaT1 urothelial cell carcinoma of the bladder. *J. Clin. Pathol.* **59**, 83–88 (2006).
97. Margulis, V., Shariat, S. F., Ashfaq, R., Sagalowsky, A. I. & Lotan, Y. Ki-67 is an independent predictor of bladder cancer outcome in patients treated with radical cystectomy for organ-confined disease. *Clin. Cancer Res.* **12**, 7369–7373 (2006).
98. Margulis, V. *et al.* Multi-institutional validation of the predictive value of Ki-67 labeling index in patients with urinary bladder cancer. *J. Natl Cancer Inst.* **101**, 114–119 (2009).
99. Ramos, D. *et al.* Prognostic value of morphometry in low grade papillary urothelial bladder neoplasms. *Anal. Quant. Cytol. Histol.* **26**, 285–294 (2004).
100. Ali-El-Dein, B. *et al.* Superficial bladder tumours: analysis of prognostic factors and construction of a predictive index. *BJU Int.* **92**, 393–399 (2003).
101. Loughman, N. T., Lin, B. P., Dent, O. F. & Newland, R. C. DNA ploidy of bladder cancer using bladder biopsy supernate specimens. *Anal. Quant. Cytol. Histol.* **25**, 146–158 (2003).
102. Baak, J. P. *et al.* DNA cytometric features in biopsies of TaT1 urothelial cell cancer predict recurrence and stage progression more accurately than stage, grade, or treatment modality. *Urology* **61**, 1266–1272 (2003).
103. Bol, M. G. *et al.* Correlation of grade of urothelial cell carcinomas and DNA histogram features assessed by flow cytometry and automated image cytometry. *Anal. Cell. Pathol.* **25**, 147–153 (2003).
104. Bellaoui, H. *et al.* Flow cytometric DNA analysis and cytology in diagnosis and prognosis of bladder tumors: preliminary results of a comparative study of bladder lavage. *Ann. Urol. (Paris)* **36**, 45–52 (2002).
105. Caraway, N. P., Khanna, A., Payne, L., Kamat, A. M. & Katz, R. L. Combination of cytologic evaluation and quantitative digital cytometry is reliable in detecting recurrent disease in patients with urinary diversions. *Cancer* **111**, 323–329 (2007).
106. Falkman, K., Tribukait, B., Nyman, C. R., Larsson, P. & Norming, U. S-phase fraction in superficial urothelial carcinoma of the bladder—a prospective, long-term, follow-up study. *Scand. J. Urol. Nephrol.* **38**, 278–284 (2004).
107. Lin, J. *et al.* E-cadherin promoter polymorphism (C-160A) and risk of recurrence in patients with superficial bladder cancer. *Clin. Genet.* **70**, 240–245 (2006).
108. Palit, V., Phillips, R. M., Puri, R., Shah, T. & Bibby, M. C. Expression of HIF-1alpha and Glut-1 in human bladder cancer. *Oncol. Rep.* **14**, 909–913 (2005).
109. Chai, C. Y. *et al.* Hypoxia-inducible factor-1alpha expression correlates with focal macrophage infiltration, angiogenesis and unfavourable prognosis in urothelial carcinoma. *J. Clin. Pathol.* **61**, 658–664 (2008).
110. Crew, J. P. *et al.* Urinary vascular endothelial growth factor and its correlation with bladder cancer recurrence rates. *J. Urol.* **161**, 799–804 (1999).
111. Crew, J. P. *et al.* Vascular endothelial growth factor is a predictor of relapse and stage progression in superficial bladder cancer. *Cancer Res.* **57**, 5281–5285 (1997).
112. Turner, K. J. *et al.* The hypoxia-inducible genes VEGF and CA9 are differentially regulated in superficial versus invasive bladder cancer. *Br. J. Cancer* **86**, 1276–1282 (2002).
113. Tickoo, S. K. *et al.* Hypoxia-inducible factor and mammalian target of rapamycin pathway markers in urothelial carcinoma of the bladder: possible therapeutic implications. *BJU Int.* **107**, 844–849 (2011).
114. Platt, F. M. *et al.* Spectrum of phosphatidylinositol 3-kinase pathway gene alterations in bladder cancer. *Clin. Cancer Res.* **15**, 6008–6017 (2009).
115. Schultz, L. *et al.* Expression status and prognostic significance of mammalian target of rapamycin pathway members in urothelial carcinoma of urinary bladder after cystectomy. *Cancer* **116**, 5517–5526 (2010).
116. Comperat, E. *et al.* Aurora-A/STK-15 is a predictive factor for recurrent behaviour in non-invasive bladder carcinoma: a study of 128 cases of non-invasive neoplasms. *Virchows Arch.* **450**, 419–424 (2007).
117. Mhawech-Fauceglia, P., Fischer, G., Beck, A., Cheney, R. T. & Herrmann, F. R. Raf1, Aurora-A/STK15 and E-cadherin biomarkers expression in patients with pTa/pT1 urothelial bladder carcinoma; a retrospective TMA study of 246 patients with long-term follow-up. *Eur. J. Surg. Oncol.* **32**, 439–444 (2006).
118. Veerla, S., Panagopoulos, I., Jin, Y., Lindgren, D. & Hoglund, M. Promoter analysis of epigenetically controlled genes in bladder cancer. *Genes Chromosomes Cancer* **47**, 368–378 (2008).
119. Wszolek, M. F. *et al.* A MicroRNA expression profile defining the invasive bladder tumor phenotype. *Urol. Oncol.* http://dx.doi.org/10.1016/j.urolonc.2009.08.024 (2009).
120. Catto, J. W. *et al.* Distinct microRNA alterations characterize high- and low-grade bladder cancer. *Cancer Res.* **69**, 8472–8481 (2009).
121. Iyer, G., Milowsky, M. I. & Bajorin, D. F. Novel strategies for treating relapsed/refractory urothelial carcinoma. *Expert Rev. Anticancer Ther.* **10**, 1917–1932 (2010).
122. Wallerand, H., Reiter, R. R. & Ravaud, A. Molecular targeting in the treatment of either advanced or metastatic bladder cancer or both according to the signalling pathways. *Curr. Opin. Urol.* **18**, 524–532 (2008).
123. Amit, D., Tamir, S., Birman, T., Gofrit, O. N. & Hochberg, A. Development of targeted therapy for bladder cancer mediated by a double promoter plasmid expressing diphtheria toxin under the control of IGF2-P3 and IGF2-P4 regulatory sequences. *Int. J. Clin. Exp. Med.* **4**, 91–102 (2011).
124. Gerullis, H. *et al.* Long-term response in advanced bladder cancer involving the use of temsirolimus and vinflunine after platin resistance. *Anticancer Drugs* **22**, 940–943 (2011).
125. Yafi, F. A., North, S. & Kassouf, W. First- and second-line therapy for metastatic urothelial carcinoma of the bladder. *Curr. Oncol.* **18**, e25–34 (2011).
126. Zhang, X. & Godbey, W. T. Preclinical evaluation of a gene therapy treatment for transitional cell carcinoma. *Cancer Gene Ther.* **18**, 34–41 (2011).
127. Smaldone, M. C. & Davies, B. J. BC-819, a plasmid comprising the H19 gene regulatory sequences and diphtheria toxin A, for the potential targeted therapy of cancers. *Curr. Opin. Mol. Ther.* **12**, 607–616 (2010).
128. Kramer, M. W., Krege, S., Peters, I., Merseburger, A. S. & Kuczyk, M. A. Targeted therapy of urological tumours. Experimental field or established therapeutic approach? *Urologe A.* **49**, 1260–1265 (2010).
129. Ching, C. B. & Hansel, D. E. Expanding therapeutic targets in bladder cancer: the PI3K/Akt/mTOR pathway. *Lab. Invest.* **90**, 1406–1414 (2010).
130. Black, P. C., Agarwal, P. K. & Dinney, C. P. Targeted therapies in bladder cancer—an update. *Urol. Oncol.* **25**, 433–438 (2007).
131. Black, P. C. & Dinney, C. P. Bladder cancer angiogenesis and metastasis–translation from murine model to clinical trial. *Cancer Metastasis Rev.* **26**, 623–634 (2007).
132. Downward, J. Targeting RAS signalling pathways in cancer therapy. *Nat. Rev. Cancer* **3**, 11–22 (2003).
133. Bellmunt, J., Hussain, M. & Dinney, C. P. Novel approaches with targeted therapies in bladder cancer. Therapy of bladder cancer by blockade of the epidermal growth factor receptor family. *Crit. Rev. Oncol. Hematol.* **46**, S85–S104 (2003).
134. Wallerand, H., Robert, G., Bernhard, J. C., Ravaud, A. & Ferriere, J. M. Targeted therapy for locally advanced and/or metastatic bladder cancer. *Prog. Urol.* **18**, 407–417 (2008).
135. Hussain, M. H. *et al.* Trastuzumab, paclitaxel, carboplatin, and gemcitabine in advanced human epidermal growth factor receptor-2/neu-positive urothelial carcinoma: results of a multicenter phase II National Cancer Institute trial. *J. Clin. Oncol.* **25**, 2218–2224 (2007).
136. Hansel, D. E., Swain, E., Dreicer, R. & Tubbs, R. R. HER2 overexpression and amplification in urothelial carcinoma of the bladder is associated with MYC coamplification in a subset of cases. *Am. J. Clin. Pathol.* **130**, 274–281 (2008).
137. Inoue, K. *et al.* Paclitaxel enhances the effects of the anti-epidermal growth factor receptor monoclonal antibody ImClone C225 in mice with metastatic human bladder transitional cell carcinoma. *Clin. Cancer Res.* **6**, 4874–4884 (2000).
138. Perrotte, P. *et al.* Anti-epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. *Clin. Cancer Res.* **5**, 257–265 (1999).
139. Philips, G. K. *et al.* A phase II trial of cisplatin, fixed dose-rate gemcitabine and gefitinib for advanced urothelial tract carcinoma: results of the Cancer and Leukaemia Group B 90102. *BJU Int.* **101**, 20–25 (2008).
140. Philips, G. K. *et al.* A phase II trial of cisplatin (C), gemcitabine (G.) and gefitinib for advanced urothelial tract carcinoma: results of Cancer and Leukemia Group B (CALGB) 90102. *Ann. Oncol.* **20**, 1074–1079 (2009).
141. Wulfing, C. *et al.* A single-arm, multicenter, open-label phase 2 study of lapatinib as the second-line treatment of patients with locally advanced or metastatic transitional cell carcinoma. *Cancer* **115**, 2881–2890 (2009).
142. Bradley, D. A. *et al.* Randomized, double-blind, placebo-controlled phase II trial of maintenance sunitinib versus placebo after chemotherapy for patients with advanced urothelial carcinoma: scientific rationale and study design. *Clin. Genitourin. Cancer* **5**, 460–463 (2007).

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143. Hahn, N. M. *et al.* Phase II trial of cisplatin, gemcitabine, and bevacizumab as first-line therapy for metastatic urothelial carcinoma: Hoosier Oncology Group GU 04–75. *J. Clin. Oncol.* **29**, 1525–1530 (2011).
144. Elfiky, A. A. & Rosenberg, J. E. Targeting angiogenesis in bladder cancer. *Curr. Oncol. Rep.* **11**, 244–249 (2009).
145. Miyamoto, H. *et al.* Non-invasive papillary urothelial neoplasms: the 2004 WHO/ISUP classification system. *Pathol. Int.* **60**, 1–8 (2010).
146. Millan-Rodriguez, F., Chechile-Toniolo, G., Salvador-Bayarri, J., Palou, J. & Vicente-Rodriguez, J. Multivariate analysis of the prognostic factors of primary superficial bladder cancer. *J. Urol.* **163**, 73–78 (2000).
147. Soloway, M. S., Sofer, M. & Vaidya, A. Contemporary management of stage T1 transitional cell carcinoma of the bladder. *J. Urol.* **167**, 1573–1583 (2002).
148. Bensalah, K., Montorsi, F. & Shariat, S. F. Challenges of cancer biomarker profiling. *Eur. Urol.* **52**, 1601–1609 (2007).

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